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Taxonomic study on Japanese *Salvia* (Lamiaceae): Phylogenetic position of *S. akiensis*, and polyphyletic nature of *S. lutescens* var. *intermedia*

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Abstract

Both *Salvia akiensis* and *S. lutescens* (Lamiaceae) are endemic to Japan. *Salvia akiensis* was recently described in 2014 in the Chugoku (= SW Honshu) region, and each four varieties of *S. lutescens* distributed allopatrically. Among varieties in *S. lutescens*, var. *intermedia* show a disjunctive distribution in the Kanto (=E Honshu) and Kinki (= W Honshu) regions. Recent field studies of *S. lutescens* var. *intermedia* revealed several morphological differences between the Kanto and Kinki populations. Here, I evaluated these differences among *Salvia lutescens* var. *intermedia* and its allies with morphological analysis and molecular phylogenetic analyses of nuclear ribosomal DNA (internal and external transcribed spacer regions) and plastid DNA (*ycf1-rps15* spacer, *rbcL*, and *trnL-F*) sequences. Both morphological analysis and molecular phylogenetic analyses showed that *S. lutescens* var. *intermedia* from the Kinki region and var. *lutescens* were closely related to each other. However, var. *intermedia* from the Kanto region exhibited an association with *S. lutescens* var. *crenata* and var. *stolonifera*, which also grew in eastern Japan, rather than var. *intermedia* in the Kinki region. These results indicated that *S. lutescens* var. *intermedia* is not a taxon with a disjunctive distribution, but a combination of two or more allopatric taxa. Present study also suggested that *S. akiensis* was most closely related to *S. omerocalyx*.

Keywords

cpDNA, Lamiaceae, nrDNA, Phylogenetics, *Salvia akiensis*, *Salvia lutescens*

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Introduction

The genus *Salvia* L. (tribe Mentheae) is the largest genus in Lamiaceae; it comprises nearly 1,000 species. *Salvia* has radiated extensively into three regions of the world: Central and South America (500 spp.), West Asia (200 spp.), and East Asia (100 spp.) (Alziar, 1988–1993). In Japan, twelve species, eight varieties, and one putative hybrid have been described since Thunberg's (1784) first account. The genus was classified into three subgenera (subg.), including *Allagospadonopsis* Briq., *Salvia*, and *Sclarea* (Moench) Benth, (Hihara et al. 2001, Inoue 1997, Murata and Yamazaki 1993, Takano et al. 2014). Most of the taxa are endemic to Japan, with the exception of *S. japonica* Thunb., *S. nipponica* Miq. and *S. plebeia* R. Br. (Murata and Yamazaki 1993).

There are four varieties known in *S. lutescens* (Koidz.) Koidz.: var. *crenata*, var. *intermedia*, var. *lutescens*, and var. *stolonifera* (Murata 1952, Yonekura and Kajita 2003 onwards). Fukuoka and Kurosaki (1982) noticed distribution of each taxon does not overlap and clarified that the distribution of var. *crenata* on the Japan Sea side of Central to Northern Honshu, var. *stolonifera* on the Pacific side of Central Honshu, var. *lutescens* around the Suzuka Mountain range (Mie Pref., W Honshu), and the disjunctive distribution of var. *intermedia* in the Kanto (E Honshu) and Kinki regions (W Honshu) based on herbarium works.

Takano and Okada (2011) conducted molecular phylogenetic analyses of Japanese *Salvia* and found that the species were distributed among three subclades: (1) *S. plebeia* (subg. *Sclarea*), (2) subg. *Salvia*, and (3) subg. *Allagospadonopsis*. They also found four varieties of *S. lutescens* that did not form a monophyletic group; instead, they were dispersed among several clades in phylogenetic trees, based on both plastid DNA (cpDNA) and nuclear ribosomal DNA (nrDNA) data, and their topologies were not concordant with each other. In addition, they became paraphyletic in the phylogenetic trees based on combined cpCNA and nrDNA data (Takano and Okada 2011). Furthermore, during a recent field survey, I noticed that *S. lutescens* var. *intermedia* in the Kanto and Kinki regions had different morphological characteristics. The basal part of the anther connective was generally glabrous in the Kanto population, but it was pilose in the Kinki population. Also, in the Kanto population, the stalk of the inflorescence declinated toward the ground after flowering, and it typically became proliferous; in contrast, in the Kinki population, the inflorescence grew erect, and it was never proliferous.

Recently, a new species of Japanese *Salvia*, *S. akiensis* A.Takano, T.Sera et Kurosaki has been described from Shimane and Hiroshima Prefectures (Takano et al. 2014). At the moment, this species shows disjunctive distribution, ca. 40 km away from each, and the habitat is also very different between Hiroshima and Shimane: it grows among bamboo by roadsides and on slopes below evergreen mixed forests and plantations in Shimane (Sakoda et al. 2014), but it is found in moist, shallow soil on rock walls by streams in deciduous forests in Hiroshima (Takano et al. 2014). Therefore, it may wonder if the species be monophyletic. Takano et al. (2014) discussed relationships among *S. akiensis*, *S. isensis* Nakai ex H.Hara, *S. lutescens* and *S. omerocalyx* Hayata

based on morphological characters, but molecular phylogenetic position of *S. akiensis* remains unclear.

As a step toward taxonomic revision of variety of *S. lutescens* and to confirm monophyly and phylogenetic position of *S. akiensis*, morphological and molecular phylogenetic analyses were conducted. Takano and Okada (2011) followed the Murata and Yamazaki (1993) system in which treated var. *intermedia* as a forma f. *lobatocrenata* and var. *lutescens* as f. *lutescens*, however, here I follow the Murata (1952) system (=Y-list, Yonekura and Kajita 2003 onwards), and each infraspecific taxon of *S. lutescens* is treated as a variety.

Materials and methods

Morphological analyses on *S.lutescens* in herbaria

Murata (1952) studied morphological variations in the plants of subgen. *Allagospadonopsis* in Japan and found hairiness, number or shape of leaflets, presence /absence of glandular hairs were so variable and could not be used as diagnostic characters. Diagnostic characters separated each variety of *S. lutescens* are indumentums of the basal part of the anther connective and floral color (Nakai 1950, Murata 1952). Among varieties, var. *lutescens* shows pale yellow flowers and pilose at the base of anther connective, var. *intermedia* shows deep violet corolla and pilose at the base of anther connective, var. *crenata* does purple corolla and glabrous base of anther connective. Floral color and indumentums of var. *stolonifera* is same as var. *crenata*, however, var. *stolonifera* extends its stolon after anthesis (Nakai 1950). Since it is difficult to know exact floral color by examining dry specimens, the indumentums at the base of the anther connective were observed for glabrousity in selected specimens, which bore at least several flowers. A total of 89 specimens of *S. lutescens* var. *intermedia*, including its syntypes, of the 34 specimens are from Kanto region and 55 from Kinki, were examined in the following herbaria: the Museum of Nature and Human Activities, Hyogo (HYO); the Kanagawa Prefectural Museum (KPM); Kyoto University (KYO); Tokyo Metropolitan University (MAK), the Osaka Museum of Natural History (OSA), and The University of Tokyo (TI) (Appendix 1). Additionally, all the specimens of *S. lutescens* var. *lutescens* including its holotype at KYO were examined on the same characters, since no information on that character is available.

DNA extraction, PCR, and DNA sequencing

The protocols for DNA extraction, polymerase chain reaction (PCR), purification, and DNA sequencing were described previously by Takano and Okada (2011). The PCR conditions and the PCR and sequencing primers for *rbcL*, the *trnL-F* intergenic spacer region of cpDNA (*trnL-F*), and the internal transcribed spacer (ITS) region of nuclear

ribosomal DNA (nrDNA) were also described previously by Takano and Okada (2011). To amplify in the *ycf1-rps15* spacer region found in cpDNA (*ycf1-rps15*), *5711f* and *rps15r* (both from Drew and Sytsma 2011) were used as PCR primers in PCR assays, and ETS-*bdf1* (Drew and Sytsma 2011) and 18S-E (Baldwin and Markos 1998) were used to amplify the external transcribed spacer (ETS) sequence from 18S-26S ribosomal DNA. The four PCR primers were also used for sequencing. The PCR conditions for amplifying the two loci were: denaturation at 95 °C for 3 min, followed by 40 cycles at 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 5 min.

Sequence alignment and phylogenetic analysis

Raw sequence data were assembled and edited manually, with BioEdit software (ver. 7.2.5 Hall 1999)

DNA sequences were aligned with the CLUSTALW 1.83 software package, with default settings and multiple alignments (Thompson et al. 1994). Alignments of the *rbcL*, *trnL-F*, and *ycf1-rps15* sequences of cpDNA, and the ITS and ETS sequences of nrDNA were combined. Gaps were deleted.

Compared to Takano and Okada (2011), the ETS (Baldwin and Markos 1998) and *ycf1-rps15* of cpDNA (Dong et al. 2015) were newly sequenced for all samples. Further, two individuals of *S. akiensis* and three of *S. lutescens* var. *intermedia*, three of *S. lutescens* var. *crenata*, and one each of *S. isensis*, *S. japonica* var. *japonica*, *S. lutescens* var. *lutescens*, and *S. plebeia* were newly added for the analysis. The sampling sites of *S. lutescens* group were shown in Fig. 1. A total of 36 individuals of *Salvia* were used, including all the *Salvia* taxa from Japan and one Taiwanese *Salvia* (*S. arisanensis* Hayata). *Salvia polystachya* M. Martens et Galeotti and *S. plebeia* were selected as outgroup; the former species belonged to clade II sensu Maria and Classen-Bockhoff (2014), which became a sister to group IV and contained the East Asian *Salvia*; the latter species became a sister to a species of the subgenus *Allagospadonopsis* and *Salvia* (Hu 2015). Materials, accession numbers for the sequences, vouchers, and references to the literature are presented in Table 1. The sampling sites for the varieties of *S. lutescens* are shown in Fig. 1.

The incongruence length difference (ILD) test (Farris et al. 1994) was used to evaluate congruence between the chloroplast and the nuclear data sets. 100 replications were performed using PAUP*4.010b (Swofford 2002). As the ILD test ($P < 0.01$) suggested incongruence between the two datasets, and the topologies also exhibited discordance, I performed separate analyses for the cpDNA and the nrDNA data. Maximum Likelihood (ML) and Bayesian inference (BI) were used. Nucleotide substitution model parameters were determined for each partition by gene was evaluated with KAKUSAN 4.0 (Tanabe 2007), and the corrected Akaike information criterion (AICc) (Sugiura 1978) was used for model selection. For the cpDNA partitions KAKUSAN suggested the HKY85 (*rbcL*) and GTR+G (trn*L-F*, *ycf1-rps15*spacer) models, and the HKY85 model for ETS and GTR+G model for ITS for the nrDNA partitions. The ML

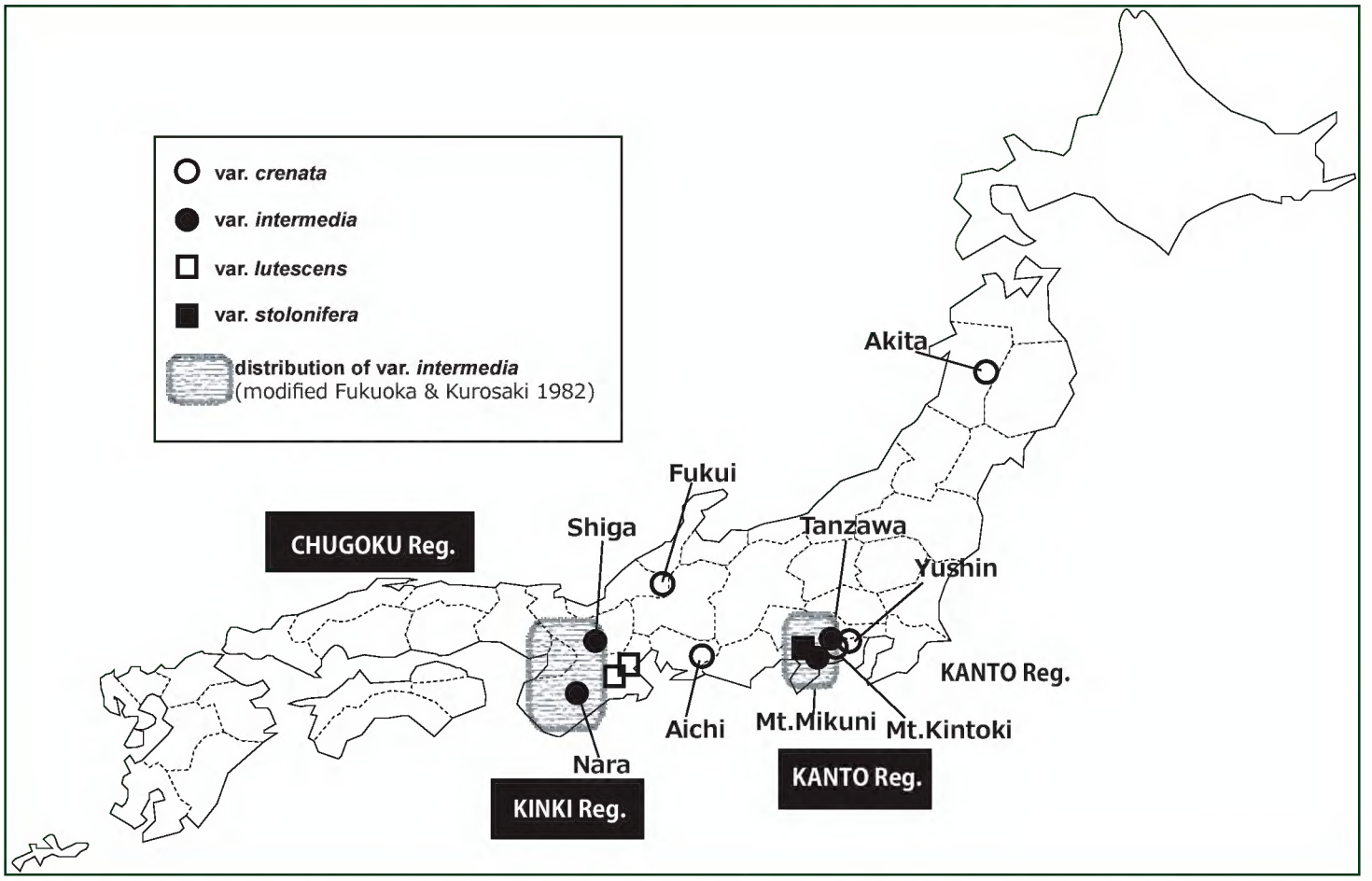

Figure 1. Map of Japan shows the sites where *Salvia lutescens* taxa were sampled. Open circle = var. *crenata*, filled circle = var. *intermedia*, open square = var. *lutescens*, filled square = var. *stolonifera*. The areas encircled with dotted lines show the Kinki and Kanto regions, as indicated.

analyses were completed using TREEFINDER version March 2011 (Jobb et al. 2004). A replicated (500 iterations) partitioned analysis was performed with bootstrap (1000 rounds) using AICc separated model for nrDNA dataset and AICc proportional model for cpDNA dataset. Bayesian evolutionary analysis using partitioned datasets were run in BEAST v.1.8.3 (Drummond et al. 2012, Heled and Drummond 2010) with 20 million Markov Chain Monte Carlo (MCMC) iterations, under an uncorrelated relaxed clock (Drummond et al. 2006), Yule process of speciation with a random starting tree for each partition. Convergence of the chains was checked using the program Tracer 1.6 (Rambaut et al. 2014). High effective sample sizes were observed for all parameters (posterior ESS values > 200 for the combined analyses). Maximum clade credibility trees with divergence times means and 95% highest probability densities (HPDs) were produced using Tree Annotator (Drummond et al.2012).

Results

Morphological characteristics

Among the 89 specimens of *S. lutescens* var. *intermedia* examined, 52 specimens from the Kinki region were pilose at the base of the anther connective (Fig. 2), and no speci-

Table 1. Taxa, Genbank accession number, and voucher specimens/references used in this study. Newly sequecned data are shown bold.

Name	Pop. Code	*rbcL*	*trnL-F*	*ycf1-rps15*	ETS	ITS	Voucher / References
S. akiensis A.Takano, T.Sera et Kurosaki	HIR (Hirohsima Pref.)	LC124176	LC124188	LC060529	LC060825	LC060729	A.Takano and N.Kurosaki with T.Sera 130606-1(HYO)
	S1(Shimane Pref.)	LC124177	LC124189	LC060530	LC060826	LC060728	M.Sakoda et al. 1 (HYO,KYO)
S. arisanensis Hayata		AB295063	AB295074	LC060531	LC060827	AB295085	Sudarmono and Okada (2007)
S. glabrescens (Franch. et Sav.) Makino							
var. *glabrescens*	FS (Wakasa, Fukui)	AB541134	AB541148	LC060532	LC060828	AB541120	Takano and Okada (2011)
var. *repens* (Koidz.) Kurosaki	KY (Kyoto)	AB295064	AB295075	LC060533	LC060829	AB295086	Sudarmono and Okada (2007)
S. isensis Nakai ex Hara	MIE	AB266221	AB266231	LC060534	LC060830	AB266241	Sudarmono and Okada (2007)
	AICHI	LC124178	LC124190	LC060535	LC060831	LC060730	A-200933 (living material at Hiroshima Bot.Gard. Originally from Owariasahi city, Aichi Pref.)
S. japonica Thunb.							
f. *albiflora* Hiyama		AB266220	AB266230	LC060536	LC060832	AB260240	Sudarmono and Okada (2007)
f. *japonica*	Osaka	AB266219	AB266229	LC060537	LC060833	AB266239	Sudarmono and Okada (2007)
f. *japonica*	Gotenba	LC124179	LC124191	LC060538	LC060834	LC060731	A.Takano 140806-5 (HYO)
f. *longipes* (Nakai) Sugimoto		AB266218	AB266228	LC060539	LC060835	AB266238	Sudarmono and Okada (2007)
S. koyamae Makino		AB541128	AB541142	LC060540	LC060836	AB541114	Takano and Okada (2011)
S. lutescens Koidz.							
var. *crenata* (Makino) Murata	AICHI	AB266223	AB266233	LC060541	LC060837	AB266243	Sudarmono and Okada (2007)
	Yushin	AB353202	AB353198	LC060542	LC060838	AB353206	Takano and Okada (2011)
	Akita	LC124180	LC124193	LC124205	LC124201	LC124203	Y. Horhii, S. Nishida et al. 2015026 (HYO)
	Fukui	LC124181	LC124194	LC124204	LC124200	LC124202	A.Takano 150702-1a (HYO)
var. *intermedia* (Makino) Murata	Nara	LC124182	LC124195	LC060544	LC060840	AB295097	Sudarmono and Okada (2007)
	Shiga	LC124183	LC124196	LC060546	LC060842	LC060735	A.Takano 140821-1 (HYO)
	Mt.Mikuni	LC124184	LC124197	LC060547	LC060843	LC060733	A.Takano 140806-4 (HYO)
	Tanzawa	LC124185	LC124198	LC060548	LC060844	LC060734	A.Takano 140622-2 (HYO)
var. *lutescens* Koidz.	MIE	AB266222	AB266232	LC060549	LC060845	AB266242	Sudarmono and Okada (2007)
	Aoyama	LC124186	LC128192	LC060550	LC060846	LC060737	a201241 (living material at Hiroshima Bot.Gard. Originally from Aoyama Kogen, Mie Pref.)

Name	Pop. Code	*rbcL*	*trnL-F*	*ycf1-rps15*	ETS	ITS	Voucher / References
var. *stolonifera* G.Nakai		AB541139	AB541153	LC060551	LC060847	AB541125	Takano and Okada (2011)
S. nipponica Miq.							
var. *nipponica*	TOKU (Tokushima)	AB541132	AB541146	LC060552	LC060848	AB541118	Takano and Okada (2011)
	KUMA (Kumamoto)	AB541127	AB541141	LC060553	LC060849	AB541113	Takano and Okada (2011)
var. *kisoensis* K.Imai	NAK	AB541136	AB541150	LC060554	LC060850	AB541122	Takano and Okada (2011)
S. omerocalyx Hayata							
var. *omerocalyx*	HI (Hidaka, Hyogo)	AB353204	AB353196	LC060555	LC060851	AB353200	Takano and Okada (2011)
	HYO (Yabu, Hyogo)	AB353205	AB353197	LC060556	LC060852	AB353201	Takano and Okada (2011)
var. *prostrata* Satake		AB541138	AB541152	LC060557	LC060853	AB541124	Takano and Okada (2011)
S. pygmaea Matsum.							
var. *pygmaea*		AB295072	AB295083	LC060558	LC060854	AB295094	Sudarmono and Okada (2007)
var. *simplicior* Hatus. ex T.Yamaz.		AB541140	AB541154	LC060559	LC060855	AB541126	Takano and Okada (2011)
S. ranzaniana Makino		AB287373	AB287374	LC060560	LC060856	AB287375	Sudarmono and Okada (2007)
S. ×*sakuensis* Naruh. et Hihara		AB541129	AB541143	LC060561	LC060857	AB541116	Takano and Okada (2011)
Outgroup							
S. plebeia R.Br.	KIZU	AB295073	AB295084	LC060563	LC060858	AB295095	Sudarmono and Okada (2007)
	KAMI	LC124187	LC124199	LC060562	LC060859	LC060738	A.Takano and N.Kurosaki 090607-2 (HYO)
S. Polystachya M.Martens et Galeotti		AY570435	JF301399	JF289067	JF301334	JF301356	Drew and Systma (2011)

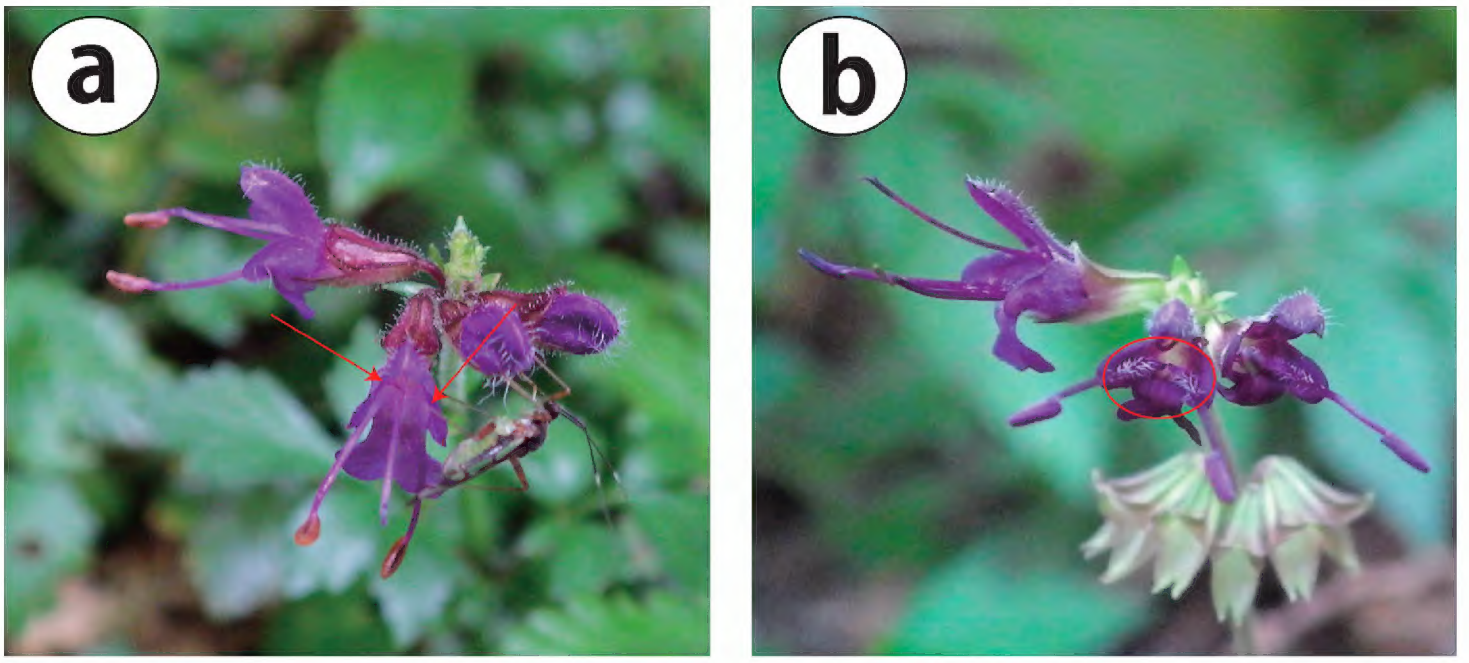

Figure 2. Photographs of *S. lutescens* var. *intermedia* flowers. **a** Flower of *A. Takano 140806-4-2* (HYO), from Mt. Mikuni, Susono-shi, Shizuoka Pref. (Kanto region). Arrows indicate the base of the anther connective. No hairs are visible **b** Flower of *A. Takano 140813-1* (HYO), from Mt. Yamatokatsuragi, Gose-shi, Nara Pref. (Kinki region). The red open circle indicates the base of the anther connective. White hairs are visible.

mens from the Kanto region shared this characteristic (Appendix 1). Ten specimens collected from the Kanto region had at least one, but less than 10 hairs. Twenty-four specimens from the Kanto region (Fig. 2) and three specimens from the Kinki region (*Y.Kato s.n.* [KYO], *T.Kobayashi 23369* [KYO], and *A.Takano 140821-1* [HYO]) were glabrous at the base of the anther connective. However, a duplicate of *T.Kobayashi 23369* (KYO) examined at HYO was pilose at the base of the anther connective (Appendix 1).

Totally, 18 specimens of *S. lutescens* var. *lutescens* were deposited at KYO and examined, 13 of these had pilose at the base of the anther connective (Appendix 1). Four of these had no flowers, and only one specimen, *M.Hara s.n.*, collected from Mt. Takami, Maze-Mura, Iinan-gun, Mie Pref. showed glabrousity.

Phylogenetic positions of Japanese taxa in the genus *Salvia*

A likelihood analysis using the concatenate cpDNA datasets (*rbcL+trnL-F+ycf1-rps15* spacer) for 36 individuals in 23 taxa resulted in a ML tree with –lnL = 5295.264. The ML and Bayesian trees had similar topology; the Bayesian maximum clade credibility tree is shown with ML bootstrap (ML-BS) and Bayesian posterior probability (BI-PP) in Figure 4. The Japanese and Taiwanese species of subg. *Allagospadonopsis* formed a well supported clade (ML-BS/BI-PP, 100/0.97). Two subclades were found in the subg. *Allagospadonopsis* clade: (1) *S. japonica* + *S. pygmaea* + one *S. akiensis* + *S. arisanensis* + five individuals of *S. lutescens* in E Japan subclade, and (2) one *S. akiensis* (S1), two *S. isensis*, *S. lutescens* in Kinki + *S. ranzaniana* + two *S. lutescens* in the Kanto region

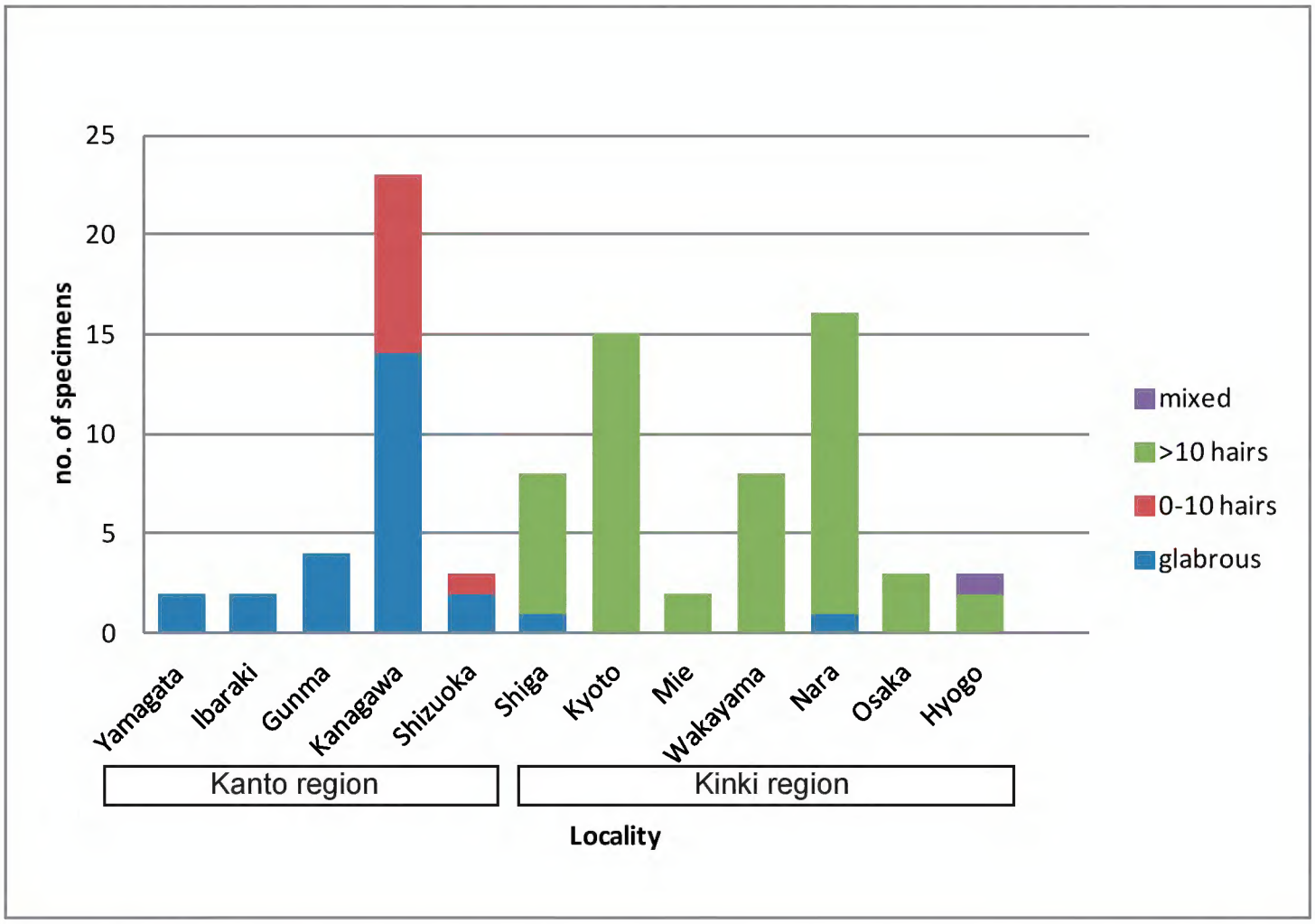

Figure 3. A graph shows the number of specimens examined indumentums at the base of anther connective.

+ *S. omerocalyx*. The latter group of taxa, minus the *S. omerocalyx* (HYO), consisted of a strongly supported subclade, with high ML-BS/BI-PP values (98/0.99). *S. lutescens* in E Japan were scattered between both subclades, but the *S. lutescens* in the Kinki region consisted a cluster with the weak support (--/0.70).

A concatenate nrDNA datasets (ETS+ITS) yielded a ML tree with –lnL = 3789.114. The ML and Bayesian trees had similar topology; the Bayesian maximum clade credibility tree is shown with ML-BS and BI-PP in Figure 5. The Japanese and Taiwanese species of subg. *Allagospadonopsis* formed a well supported clade (ML-BS/BI-PP, 100/1.00). There were four subclades in the *Allagospadonopsis* clade: (1) *S. lutescens* group in E Japan + *S. isensis* (ML-BS/BI-PP, --/0.69), (2) *S. lutescens* in Kinki + *S. ranzaniana* (ML-BS/BI-PP, 61/0.57), (3) *S. arisanensis* + *S. omerocalyx* + *S. akiensis* (ML-BS/BA-PP, 76/0.99), and (4) one *S. lutescens* var. *crenata* + *S. japonica* + *S. pygmaea* (ML-BS/BA-PP, 58/0.97). Thus, *Salvia lutescens* and its allies apparently became polyphyletic. *Salvia ranzaniana* became a sister group to *S. lutescens* in the Kinki region but the ML-BS /BA-PP support was weak (61/0.57). *Salvia isensis* became a sister group to *S. lutescens* in the Kanto region with strong ML-BS/BA-PP support (86/1.00). *Salvia akiensis* formed a strongly supported subclade with *S. omerocalyx* group (89/1.00).

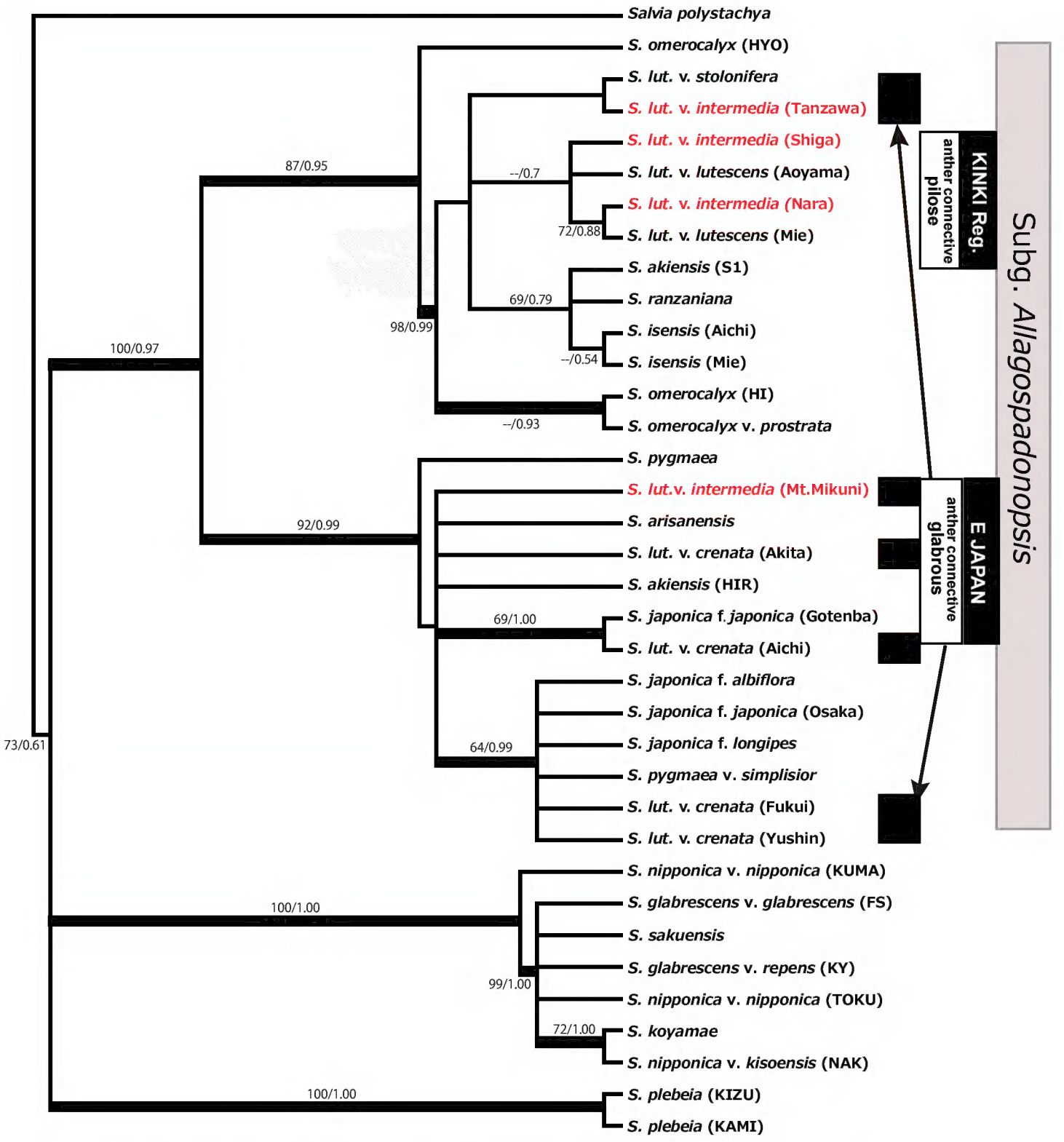

Figure 4. The Bayesian maximum clade credibility tree derived from plastid DNA (concatenate dataset of *rbcL*, trn*L-F*, *ycf1-rps15*). ML-bootstrap/Bayesian PP numbers are shown near the corresponding branch. Thick lines denote a clade that was strongly supported, with ML- bootstrap and/or Bayesian-PP greater than 95 %. ML: maximum likelihood; PP: posterior probability.

Discussion

This study suggests that *S. lutescens* var. *intermedia* is polyphyletic. Four individuals of var. *intermedia*, two from the Kanto and two from the Kinki region fell into different subclades in both molecular phylogenetic trees using cpDNA and nrDNA datasets, although the two from the Kinki region were always in the same subclade (Figs 4, 5). The plants of var. *intermedia* from the Kanto region (Tanzawa and Mt.Mikuni) fell into the same subclade in the nrDNA tree together with var. *crenata*, var. *stolonifera*,

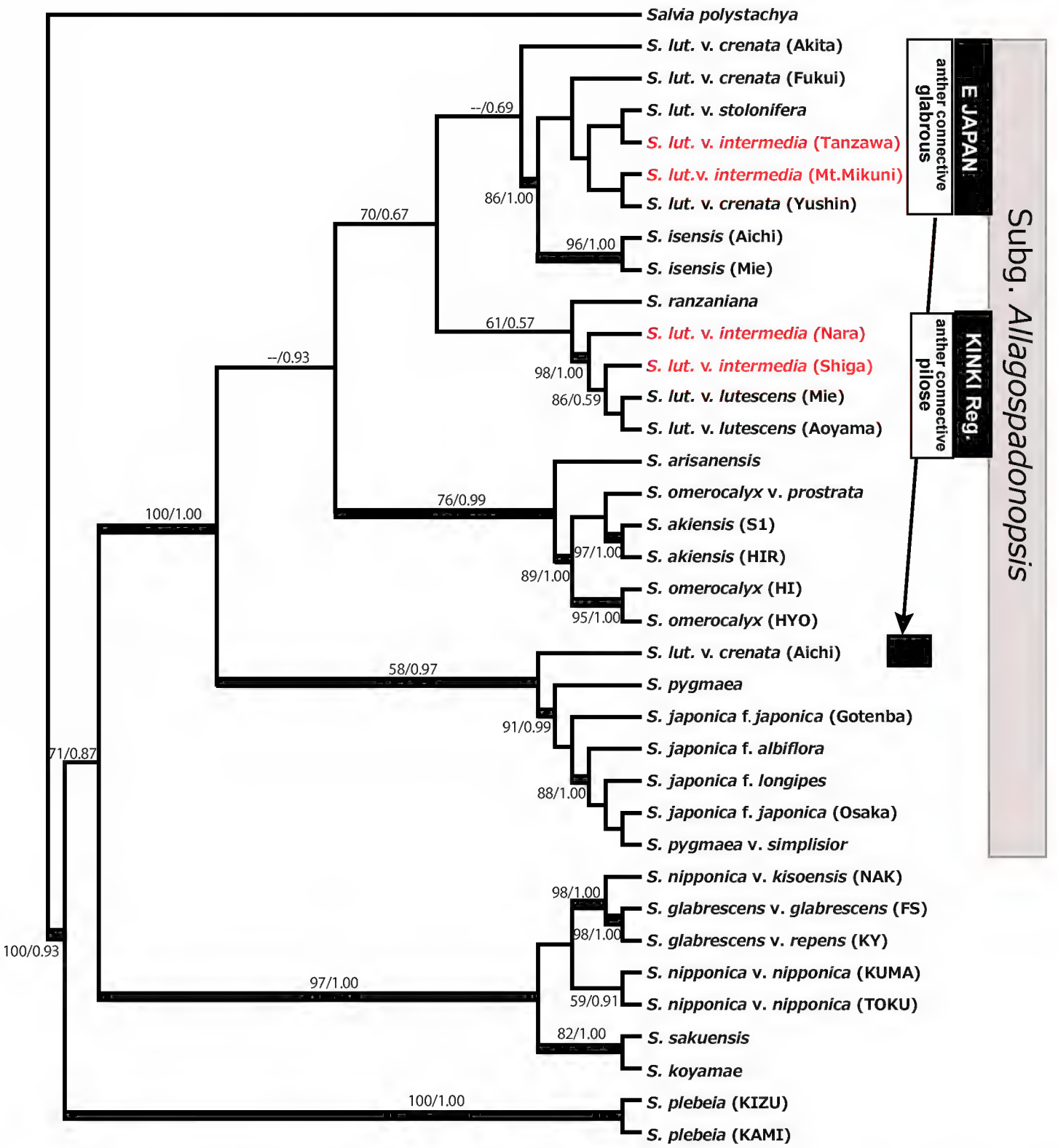

Figure 5. The Bayesian maximum clade credibility tree derived from nuclear ribosomal DNA (concatenate dataset of ETS and ITS). ML-bootstrap/Bayesian-PP numbers are shown above or below the corresponding branch. Thick lines denote a clade that was strongly supported with ML-bootstrap and/or Bayesian-PP values greater than 95 %. ML: maximum likelihood; PP: posterior probability.

and *S. isensis* whereas they fell into different subclades in the cpDNA tree. Such a contradiction might indicate that var. *intermedia* from the Kanto region have multiple origins, or might have originated via hybridization or introgressive gene flow between nighbouring taxa (e.g., Sudarmono and Okada 2007). The discordance between nr DNA and cpDNA data is common in the mint family (Trusty et al. 2004, Moon et al. 2010, Drew and Sytsma 2013, Deng et al. 2015), and chloroplast-based phylogeny often does not reflect their morphological relationships, which can be explained by

chloroplast capture (Rieseberg and Soltis 1991). Morphological analysis also supports the contention that var. *intermedia* is polyphyletic, as the specimens of var. *intermedia* studied showed in the indumentums at the base of the anther connective: pilose in the plants from the Kinki region, and glabrous in the plants from the Kanto region (Fig. 3).Therefore it is clear that var. *intermedia* from the Kinki region and the taxon from the Kanto region are different entities, suggesting that var. *intermedia* is not a taxon that shows disjunctive distribution, but is instead admixture of two or more biological entities. Additionally, as mentioned in introduction, after flowering the stalk of the inflorescence becomes declinate to ground and usually proliferous in case of the plants from the Kanto region, but never become declinate in the plants from the Kinki region. The indumentums at the base of anther connective is effective to select pollinators to avoid intrusion of insects which could not be effective pollinators (R.Classen-Bockhoff pers. Comm..) However, pollinators of var. *intermedia* in the Kinki and the Kanto region are not different (=*Bombus* (*Diversobombus*) *diversus diversus*, some Halictidae, and Syrphidae. Takano 2017). Habitat is also similar: half-shaded, on mesic soils along streamlet on the forest floor of deciduous forests. They might have begun to be diverged from each other after long geographical isolation.

On the contrary, present morphological and molecular phylogenetic analyses indicated that *S. lutescens* var. *lutescens* and var. *intermedia* from the Kinki region are closely related to each other. In molecular phylogenetic analysis, they formed a cluster in both cpDNA- and nrDNA trees, though ML-BP/BI-PP support was not strong in cpDNA tree. The morphological study revealed var. *lutescens* is pilose at the base of the anther connective: therefore, *S. lutescens* var. *intermedia* in the Kinki region share the same morphological status with var. *lutescens*. The distribution of var. *lutescens* is very near to that of var. *intermedia* in the Kinki region (Mie, Shiga, Nara Prefs.), although var. *lutescens* and populations of the Kinki regions of var. *intermedia* have never been found to grow together.

Salvia lutescens var. *intermedia* in the Kanto region may be more closely related to var. *crenata* and var. *stolonifera*. Murata (1952) mentioned that the base of anther connective is glabrous in var. *stolonifera* and var. *crenata*. The present study revealed that var. *intermedia* in the Kanto region shares this character with those two taxa. *Salvia lutescens* var. *intermedia* in the Kanto region formed a strongly supported sucblade with var. *crenata*, var. *stolonifera* and *S. isensis* in nrDNA phylogenetic tree. In the cpDNA phylogenetic tree, *S.lutescens* var.*intermedia* from the Kanto region (Mt.Mikuni) was included in the subclade containg *S. akiensis*, *S. japonica*, *S. lutescens* var. *crenata*, and *S. pygmaea* whereas *S. lutescens* var. *intermedia* (Tanzawa) formed a subclade with var. *stolonifera* and was included in the subclade containing *S. akiensis*, *S. omerocalyx*, *S. ranzaniana*, and *S.lutescens* var. *intermedia* from the Kinki + *S. isensis*. These findings suggest a close relationship among var. *crenata*, var. *stolonifera*, and var. *intermedia* from the Kanto region. Var. *intermedia* from the Kanto region may belong to var. *stolonifera* and var. *crenata*. The identity of var. *intermedia* and other varieties of *S. lutescens* are needed to be re-evaluated, and further study is necessary towards revision of varieties of *S. lutescens*.

The phylogenetic analyses also suggest that *S. akiensis* comprises a monophyletic group, as indicated by nrDNA tree, and that most of the species allied to *S. akiensis* was the *S. omerocalyx* group. *Salvia akiensis* and *S. omerocalyx* group comprised a subclade in nrDNA (ML-BS/BI-PP: 89/1.00). These two taxa did not form a subclade in cpDNA, but it may be of introgression/chloroplast capture /hybridization as mentioned above. In contrast, *S. akiensis* and *S. omerocalyx* share following characters: bearing the largest flowers among species in the subg. *Allagospadonopsis*, flower from May to June, and exhibit gynodioecy (Takano 2013; Takano et al. 2014). These characters are assumed to be symapomorph.

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Appendix 1

Specimens examined *Salvia lutescens* var. *intermedia. And* var. *lutescens.*

Salvia lutescens var. *intermedia*

Specimens glabrous at base of anther connective (27 sheets)

KANTO Region. Yamagata Pref.: Mt. Kushigata, *S.Kigawa s.n.*, July 10, 2001 (KPM *NA0151444, NA015445*). **Ibaragi Pref.:** Tsukuba, *C.Owatari s.n.*, July 8, 1895 (**syntype**, TI); Ibidem, *C.Owatari s.n.*, July 25, 1895 (**syntype**, TI). **Gunma Pref.:** Mt. Akagi, *H.Hara s.n.*, July 12, 1928 (TI); Akagi, Shikisimadori, Chubu, *H.Hara s.n.*, 11 July 1928 (TI), Kouzuke, Tone, Yunogoya-daira, *H.Hara s.n.*, 13 July, 1928(TI); Joshu, Tone-gun, Tokura, *S.Saito 145* (TI). **Kanagawa Pref.:** Hakone, *S.Ohkubo s.n.*, July 26, 1881 (**syntype** TI); Ibidem, *unknown collector*, July 26, 1881 (**syntype**, TI); ibidem, *S.Tamaki s.n.*, July 14, 1914 (TI); Minami pass, Hakone, *T.Makino 62582* (KYO); Yoduku, Yamakita-cho, *T.Katsuyama et al.*, July 23, 2005 (KPM *NA0124794*); Mikuniyama-rindo, Hakone, *Inoue et al.*, June 18, 1998 (KPM *NA0112995*); Tanzawa-Ohyama, *T.Nishio 1489* (KPM); Summit of Mt. Kintoki, Hakone, *S.Kigawa s.n.*, July 3, 1980 (KPM *NA1020531*); Ishigoya-Ochiai, Kiyokawa-mura, *H.Takahashi 20563* (KPM); Ishigoya-Ochiai, Kiyokawa-mura, *H.Takahashi 20565* (KPM); Minesaka Pass to Myojin Pass, Yamakita-cho, *S.Mori 20536* (KPM); Kaminokawa, Tsukuimachi, *S.Kigawa 20559* (KPM); Kurokura, Yamakita-machi, *M.Furuse 45371* (KPM); Ogawadani, Yamakita-cho, *A.Takano 140622-2* (HYO). **Shizuoka Pref.:** Mt.Mikuni, Fukayoshi, Susono-shi, *A.Takano 140806-4-1* (HYO); ibidem, *A.Takano 140806-4-2* (HYO).

KINKI Region. Nara Pref.; Yoshino, Yamato, *Y.Kato s.n.*, Aug. 8. 1936 (KYO). **Hyogo Pref.:**Taki-gun, Nishiki-cho, *T.Kobayashi 23369* (KYO). **Shiga Pref.:** Ikadachitochu, *A.Takano 140821-1* (HYO).

Specimens showed one to several hairs at base of anther connective (10 sheets)

KANTO Region. Pref.Sizuoka: Mt. Higane, Prov. Izu, *S. Shimazu s.n.*, July 18, 1920(KPM);

Pref.Kanagawa: Sirogane rindo, Yugawara-cho, *Y.Hasegawa 14263* (KPM); Ogawadani-rindo, Yamakita-cho, *T.Katsuyama s.n.*, Aug. 22, 1995 (KPM *NA0100397*); Marudake, Hakone, *M.Tashiro s.n.*,July 18, 1956 (KPM *NA0157166*); Tounomine, Hakone, *T.Deguchi 80495* (KPM); Tougadake, Yamakita-cho, *S.Kigawa s.n.*, July 3, 1980 (KPM *NA1020531*); Ishigoya-Ochiai, Kiyokawa-mura, *H.Takahashi 20564* (KPM), Mt.Ohmuro, Tsukui-machi, *S.Kigawa s.n.*, June 10, 1979 (KPM *NA1020566*); Hayatogawa Rindo, Tsukui-machi, *S.Kigawa 20541* (KPM), Youkizawa, Yamakitamachi, *S.Kigawa 20567* (KPM).

Specimens which showed long pilose at base of anther connective (52 sheets)

KINKI Region. Kyoto Pref.: Rakuhoku, Ohara, Otonashi W.F., *S.Hajacava s.n.*, Aug. 1896 (TI), Kyoto, Ohara, *T.Tsuyama s.n.*, Sep.7, 1934 (TI); Kiyotaki-Takao, Ukyo-ku, Kyoto, *S.Tsugaru & T.Takahashi 26448* (KYO); Mt.Hyotankuzure-yama, near Ohara, *G.Nakai 5401* (KYO); ibidem, *G.Nakai s.n.*, July 25, 1951 (KYO); Kadono-gun, Nakagawa-mura, *M.Tagawa 887* (KYO, two sheets); Maesaka Takanomine to Shimosugisaka, *S.Okamoto s.n.*, July 14, 1932 (KYO), Bodai W.F., Nakagawa, *G.Nakai 6305* (KYO); Mt. Kibune, *unknown collector*, June 28, 1921 (KYO); Kyoto-shi, Nakagawa to Bodai no Taki, *M.Hutoh 10515* (OSA); ibidem, *M.Hutoh 9264* (OSA); ibidem, *M.Hutoh 10528* (OSA); ibidem, *M.Hutoh 3465* (OSA); Mt.Hiei-san, *S.Tanaka s.n.*, June 30,1932 (OSA). **Hyogo Pref.:** Taki-gun, Nishiki-cho, *T.Kobayashi 23369* (HYO); Youtakuji, Sanda, *T.Makino 62583* (KYO); Moshi, Sanda, *A.Takano 140813-1* (HYO). **Nara Pref.:** Yamato, Sanjo-ga-dake to Gyojagaeri, *Y.Momiyama s.n.*, July 16, 1955(TI) (three sheets), Ibidem, *H.Hara s.n.*, July 16, 1955 (TI) ; ibidem, *T.Kobayashi 30611* (OSA); near the temple Kongo, Kashiwagi, Yamato, *K.Kondo s.n.*, June 8, 1928 (TI); Kosei River, Tenkawa Village, *K.Seto 44248* (OSA); Mt. Omine to Mt.Sanjogadake, *H.Hara 4683* (TI); en route from Wasamata hut to Mt.Nihon-dake, Kamikitayama-mura, *M.Okamoto 1966* (OSA); Mt.Daifugendake, *T.Kodama 10833* (OSA); Shonoiwaya-Mt.Wasamata Kamikitayama-mura, *K.Kodama 14356* (OSA); Irinami, Yamato, *S.Sakaguchi s.n.*, June, 1930 (KYO); Mt.Ohmine, *S.Sakaguchi s.n.*, Aug. 4, 1931 (KYO); enroute from Mt.Sanjo to Mt.Daihugen, *T.Kodama s.n.*, Aug.6,1959 (KYO); Mt.Sanjo, *G.Koizumi s.n.*, July 13, 1922 (KYO); Mt.Yamatokatsuragi, Gose, *A.Takano 140819-1* (HYO). **Osaka Pref.:** Mt.Izumi-katsuragi, *S.Nakanishi s.n.*, July 30, 1968 (OSA); Ibidem, *T.Nakajima s.n.*, Aug.21, 1952 (OSA); Ibidem, *C.Satonaka s.n.*, July 12, 1981 (OSA). **Wakayama Pref.:** Ryujin-Mura, Koya, *T. Nakajima s.n.*, July 31, 1931 (two sheets, TI); Doro Hacho, *G.Nakai 5213* (KYO); Ibidem, *T.Kodama s.n.*, May 30, 1951 (OSA); ibidem, *M.Hori s.n.*, May 30, 1951 (OSA); Hidaga-gun, Ooze, *S.Sakaguchi s.n.*, July 27,1932 (KYO); Mt. Sukuyama, Katsuragi-cho, Ito-gun, *K.Seto 29839* (KYO, OSA); Mt. Kurosawa, Sayiki-mura, *Y.Ogawa s.n.*, Aug.30, 1950 (KYO). **Shiga Pref.:** Otsu, *N.Takemura s.n.*, June 1901 (**Lectotype** MAK); Omi, Tochu, *M.Togashi 1205* (TI); Tochu to Ikadachi, *M.Umebayashi 737* (KYO); Mt. Hiei, *G.Murata 11415* (KYO); Ukawa, Shiga-cho, *M.Tanimoto s.n.*, June 9, 1973 (KYO); Benzaiten to Sakamoto, Mt.Hiei, S.*Tanaka s.n.*, June 30, 1932 (KYO). **Mie Pref.:** Wada, Kiwa-cho, Minami-murogun, *H.Takahashi 21040* (KYO); Taki-gun, Miyagawa-mura, Shimomate (cult.), *K.Seto 17303* (OSA).

Salvia lutescens var. *lutescens*

Specimens which showed long pilose at base of anther connective (13 sheets)

Mie Pref.: Itaya, Kata, Kameyama, *S.Tsugaru & T.Sawada 34155*(KYO); Notoyama, Suzuka-gun, *T.Hattori s.n.*, Aug. 5, 1928 (KYO); Kozu-mura, Naga-gun, *G.Nakai*

4772 (KYO);Shinzan kokuyu-rin, Iinan-gun, *Z. Tashiro s.n.*, 4.Aug., 1934 (KYO), Mt. Gozaisyo, *G.Koizumi s.n.*, 11 Jun. 1922 (KYO); Kozu-mura, Myouga-gun, (cult. at KYO) *G.Nakai 5402* (KYO); Ibidem, *G.Nakai 4773* (KYO); Nagaishi-dani, Mt. Kamagadake, Komono-cho, Mie-gun, *N. Fukuoka 4948* (KYO); Onsen-do, Mt. Gozaisho, *G.K. & S.F. s.n.*, June 1922 (KYO, holotype).

Shiga Pref.: Nasugahara, Ohara-Mura, Kouga gun, *G.Koizumi s.n.*, 2 July, 1939 (KYO), Kurotaki, Tsuchiyama-cho, Koga-gun, *T. Murase 47897* (KYO); Koga-gun, Suzuka-Pass, *H. Koyama & N. Fukuoka 55* (KYO); **Nara Pref.:** Kamide, Momomata, Mitsue-mura, Uda-gun, *K.Kawabata 9994* (KYO)